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TITLE: Characterization of a SUMO Ligase that is Essential for DNA Damage-Induced NF-κB Activation

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14. ABSTRACT

It has been recently proposed that inhibition of NF-κB may be a therapeutic target for the treatment of ER- breast cancers. As of now, the majority of NF-κB inhibitors focus on the key signal integrating complex known as the IκB kinase (IKK) complex. Since NF-κB plays a major role in many essential physiological processes in the cell, global inhibition of NF-κB at the central IKK complex could allow for increased risk of side effects as well as the desirable effects on cancer cell death. Hence, identification of specific, novel molecular targets in the NF-κB signaling pathway may lead to the identification of more specific NF-κB inhibitors. Our hypothesis is that PIASy, a SUMO ligase, is essential for DNA damage induced NF-κB activation, however is not critical for classical activation of NF-κB, leaving the more physiological pathway intact. We reveal that PIASy is signaling at the level of NEMO SUMOylation, a posttranslational modification that we recently identified being critical for DNA damage induced activation of NF-κB. Reduction of PIASy through siRNA caused inhibition of NF-κB in response to multiple DNA damaging agents commonly used in anti-cancer therapy. We provide strong evidence that PIASy is working at the level of NEMO SUMOylation and propose that PIASy is the SUMO ligase for NEMO. Furthermore, we show that the catalytic activity of PIASy is essential for NF-κB activation and hence suggest that inhibition of PIASy may be used as a more specific inhibitor in anti-cancer therapy to treat ER- breast cancer.

15. SUBJECT TERMS
PIASγ/PIASy, NEMO, NF-κB, SUMO

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Annual Summary Report

I. Introduction

Background:

Nuclear Factor-κB (NF-κB) is a transcription factor that regulates a diverse subset of genes involved in immune function, growth control, development, and regulation of apoptosis. NF-κB exists in the cell in inactive cytoplasmic complexes, with the predominant complex being p65/p50 dimers. NF-κB can be activated by a wide variety of stimuli at the cell surface such as tumor necrosis factor α (TNFα), interleukin-1 (IL-1), and lipopolysaccharide (LPS). NF-κB can also be activated in response to various DNA damaging agents, such as ionizing radiation and Topoisomerase II inhibitors (e.g. etoposide and doxorubicin/adriamycin). Activation of NF-κB in response to various DNA damaging agents is less rapid than extracellular stimuli and generally results in the activation of anti-apoptotic genes. The early key molecular events involved in NF-κB activation with various stimuli are diverse, however a key component in this signaling pathway with most stimuli is the IKK complex that is composed of three major proteins in the cytoplasm of the cell. One protein, NEMO serves as an adaptor/scaffolding component which is essential for IKK complex activity to ultimately activate NF-κB. NF-κB activation has also been suggested to play a role in resistance to therapy in advanced breast cancers. Importantly, estrogen receptor negative (ER-) breast cancers tend to have high constitutive levels of active NF-κB and activate NF-κB in response to DNA damaging anticancer agents. It has been recently proposed that inhibition of NF-κB may be a potential therapeutic target for ER- breast cancers. As of now, the majority of NF-κB inhibitors focus inhibiting the IKK complex. One potential issue with inhibiting the IKK complex is that NF-κB mediates a variety of physiologically vital processes besides those involved in the regulation of apoptosis. As a result, nonspecific inhibition of NF-κB's various target genes could allow for an increased risk of side effects and unpredictable effects on apoptosis of breast cancer cells. One solution to this dilemma is inhibition of specific NF-κB pathways upstream of IKK activation in response to DNA damaging agents. If one could selectively inhibit the NF-κB signaling pathways induced by DNA damaging anticancer agents and leave other important NF-κB signaling pathways (such as TNFα and IL-1 signaling) intact, then enhanced cancer cell death may be achieved with fewer side effects

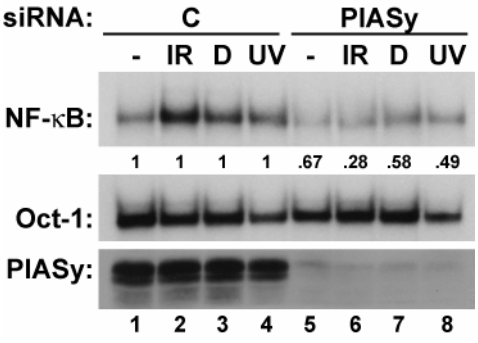
Objective/Hypothesis: PIASγ/PIASy is the SUMO ligase for NEMO and inhibition of this specific ligase may provide a novel target for chemotherapeutic treatment regimens against ER- breast cancer.

II. Body

Aim1: To understand the mechanisms governing NEMO SUMOylation

In this grant, we proposed to look at the effect of PIASy on NF-κB activation with multiple DNA damaging agents such as ionizing radiation (IR) and doxorubicin, a more commonly used agent for breast cancer treatment. We utilized PIASy siRNA in HEK 293 cells to show that PIASy expression is critical for activation of NF-κB with IR, doxorubicin, and UV (Figure 1) suggesting that PIASy is working on multiple DNA damage activators of NF-κB.

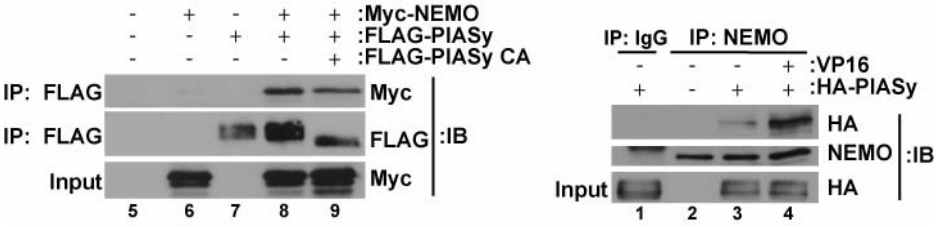
Figure 1:



HEK293 cells were transfected with control or siRNAs against different SUMO ligases. Cells were treated with 20 Gy of ionizing radiation (IR) for 90 minutes, 25 μM doxorubicin (D) for 105 minutes, and 60J/cm^2 UV for 135 minutes. Total cell extracts were made and NF-κB activity was measured using EMSA. EMSA with an Oct-1 probe was used as a control.

We also proposed to show that PIASγ/y is directly involved in NEMO SUMOylation. Note that the most common name for PIASγ is PIASy and this acronym will be used from here on. In order to show PIASy was directly involved in NEMO SUMOylation, we proposed that NEMO and PIASy could interact in an inducible manner. Instead of using an HA-PIASy construct, we used a FLAG-PIASy and the catalytically inactive PIASy construct (FLAG-PIASy CA) to test immunoprecipitation with myc-NEMO. FLAG-PIASy and the CA mutant did indeed co-immunoprecipitate myc-NEMO without stimulation (Figure 2a) suggesting that PIASy can interact with NEMO. We also proposed to do the converse immunoprecipitation, however immunoprecipiting an N-term myc-NEMO did not co-immunoprecipitate FLAG-PIASy. We believe this may be due to masking of the PIASy interaction site upon myc-NEMO immunoprecipitation. In order to get around this, we next co-immunoprecipitated a myc-tagged PIASy with endogenous NEMO bearing no tag. This interaction did occur and interestingly could be promoted further with treatment of the DNA damaging agent, etoposide (Figure 2b).

Figure 2a and b:

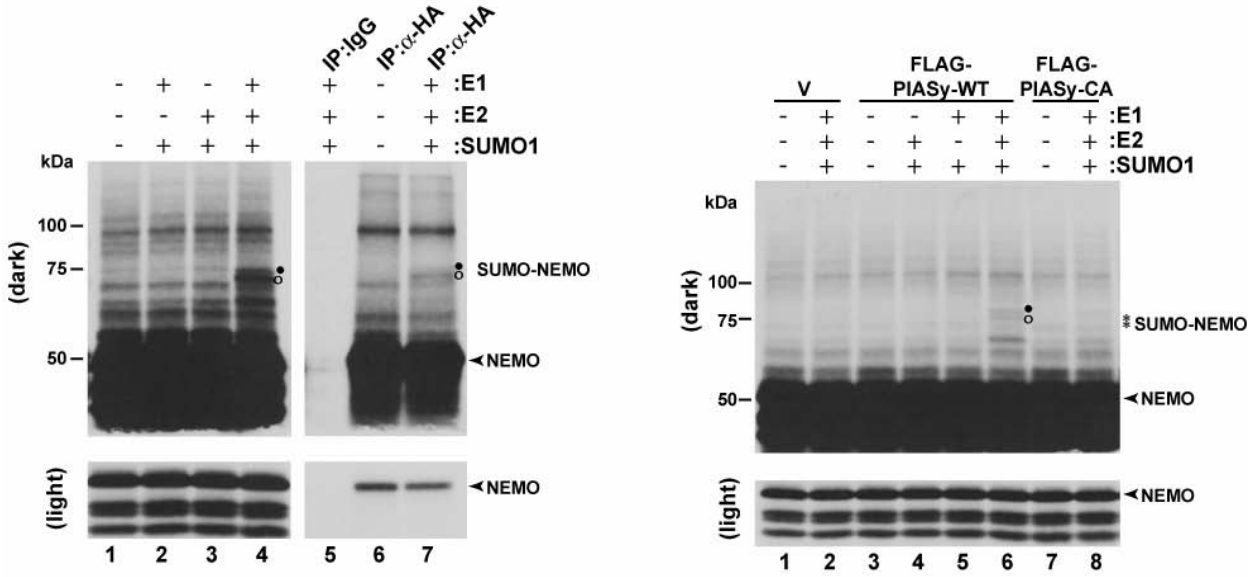


a. HEK293 cells were transfected with Flag-PIASy, Myc-NEMO, and Flag-PIASy-CA. Cells were harvested, lysed, and immunoprecipitated using anti-Flag antibody. Western blotting was performed using anti-Myc and anti-FLAG antibodies. **b.** HEK293 cells were transfected with HA-PIASy or vector alone. Lysates were immunoprecipitated anti-NEMO antibody or using a mouse IgG control. Samples were immunoblotted with anti-HA and anti-NEMO antibodies.

If PIASy was directly affecting NEMO SUMOylation, then it should be able to enhance NEMO SUMOylation in vitro. We initially proposed that we would use recombinant GST-NEMO and His-PIASy. However, we were unable to show that GST-NEMO could be SUMOylated in vitro and were unsuccessful in purifying His-PIASy with high purity and quantity. We also tried to in vitro SUMOylate recombinant His-NEMO with no success. Hence, we provided NEMO through in vitro translation of HA-NEMO in rabbit reticulocyte extracts in the presence of ^{35}S methionine. HA-NEMO was fully capable of being SUMOylated in vitro (Figure 3a). Under in vitro conditions below saturation, we were able to see that PIASy could enhance NEMO

SUMOylation that was dependent on its RING finger domain (Figure 3b) suggesting that PIASy can directly SUMOylate NEMO. We are currently in the process of using highly purified recombinant PIASy to show NEMO enhancement and trying to determine why recombinant NEMO cannot be SUMOylated in vitro.

Figure 3:



a. *In vitro* translated ^{35}S HA-NEMO was added to components for *in vitro* SUMOylation. The mixture was incubated at 30^{o}C for 150 minutes and terminated in 2xSDS sample buffer. Samples were run on an SDS-PAGE gel, fixed, and exposed to film. Alternatively, reaction products were immunoprecipitated with an anti-HA antibody and analyzed as stated above. **b.** *In vitro* SUMOylation assay was performed as stated in a, except HEK293 cells were transfected with Flag-PIASy-WT and -CA constructs. Cell lysates were immunoprecipitated with an anti-Flag antibody and washed extensively using high salt buffer. Immune purified beads were then added to the *in vitro* SUMOylation assay and incubated at 30^{o}C for 90 min. Samples were terminated and analyzed as stated in a.

Aim2: Determine if PIASγ/y dictates the ability of breast cancer cells to activate NF-κB basally and in response to DNA damaging agents.

We initially proposed that PIASy expression levels may dictate the ability of breast cancer cells to activate NF-κB and have high constitutive NF-κB activity. We initially proposed isolating mRNA from various ER+ and ER- breast cancer cell lines to assess the levels of PIASy. At the time of this proposal, a commercially reliable PIASy antibody was not available. However, we have recently been able to obtain human PIASy antibody from Dr. Mary Dasso at the NIH. We have tested this antibody and it gives strong signal (see figure 1). As a result, we will use this PIASy antibody for protein profiling of various breast cancer cell lines. We currently have cellular extracts from both ER positive and negative breast cancer cell lines and predict that PIASy levels may dictate the ability for these cells to activate NF-κB in response to DNA damage. We also proposed that PIASy could determine the sensitivity of ER+ cell line MDA-MB-231. We initially proposed to perform stable knockdown of PIASy in this cell line, however we have been able to obtain an instrument from Amaxa that gives 80% transfection efficiency in this cell line. Thus, we will utilize PIASy siRNA in these cell lines to examine their effects on basal NF-κB activation and activation in response to DNA damaging agents. We have already shown that PIASy siRNA not only blocks DNA damage induced NF-κB activation in HEK 293

cells, but also blocks this activity in HeLa (epithelial adenocarcinoma) and CEM (acute lymphoblastic leukemia) cell lines. Hence, we believe that the MDA-MB-231 cell lines will behave in a similar manner.

III. Key Research Accomplishments and Conclusions:

1. Interaction of PIASy and NEMO was demonstrated and the interaction was further induced with the DNA damaging agent, etoposide.
2. Direct ability of PIASy to enhance NEMO SUMOylation through in vitro SUMOylation assays.
3. PIASy RING finger mutant is required for NEMO SUMOylation enhancement in vitro.
4. Obtaining a PIASy antibody for analysis of ER+ and ER- breast cancer cell lysates
5. Ability to transfect ER- MDA-MB-231 cell line with high efficiency.

IV. Reportable Outcomes

1. *Manuscript:* PIASy MEDIATES NEMO SUMOYLATION AND NF-κB ACTIVATION IN RESPONSE TO GENOTOXIC STRESS, submitted and currently under revision
2. *Abstract:* A SUMO LIGASE FOR NEMO, Ubiquitin Family Meeting, 2005
3. *Presentation:* REGULATION OF NEMO SUMOYLATION AND DNA DAMAGE INDUCED NF-κB ACTIVATION BY THE SUMO LIGASE PIASY, Third International Ubiquitin Conference, 2006

V. Conclusions

Aim1: We have shown that PIASy and NEMO can interact inducibly. We have also shown that PIASy can enhance NEMO SUMOylation in vitro which is dependent on its catalytic activity. We will continue to work on experiments that have been outlined in the approved statement of work. Since we now have a PIASy antibody, we intend to look at endogenous interaction of PIASy and NEMO in response to multiple genotoxic stresses, not only etoposide. We are also trying to perform a "true" in vitro assay with NEMO and PIASy using recombinant NEMO and PIASy. However, we are currently examining what prevents recombinant NEMO from being SUMOylated in vitro. We believe that perhaps another modification on NEMO is required for its SUMOylation. We have obtained pure recombinant PIASy and are testing its ability to enhance NEMO SUMOylation.

Aim2: We have now obtained a working PIASy antibody and intend to analyze protein lysates from ER+ and ER- breast cancer cell lines available in the lab. We feel that analyzing protein levels instead of mRNA will give us a better indication if PIASy levels dictate a cell's ability to activate NF-κB both constitutively and in response to genotoxic stress. We have optimized transfection efficiency in MDA-MB-231 ER- cell lines and intend to look at modulating PIASy expression levels with constructs and siRNA to look at NF-κB effects and sensitivity to anti-cancer agents such as doxorubicin. We still intend to look at the effects of antiestrogens, such as tamoxifin on regulating PIASy levels. We also intend to look at cancer cell susceptibility with varying PIASy expression levels in ER- cell lines.

Appendices: NONE